

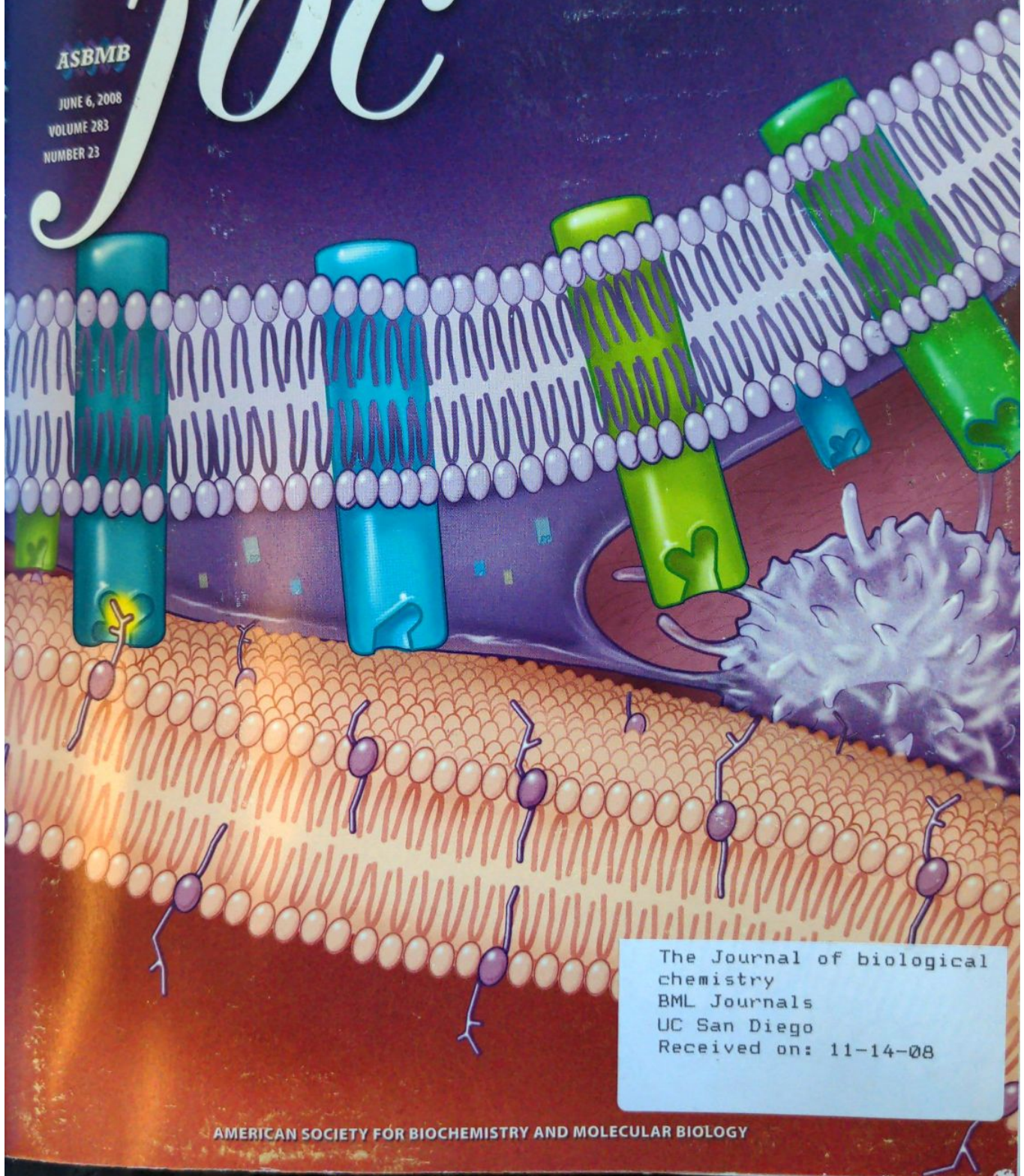
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On The Cover

Recent studies reveal that numerous oxidized phospholipids within senescent or apoptotic cell membranes adopt conformations in which the oxidized fatty acids protrude into the aqueous phase, forming a sort of "chemical braille" that can be read by multiple distinct macrophage receptors. The image illustrates a macrophage pseudopod probing the surface of an apoptotic or senescent cell that has "sprouted whiskers" in the form of a variety of oxidized fatty acid species. The generalized phenomenon of oxidized phospholipids adopting these unique conformations within membranes is described in the Lipid Whisker Model of Cell Membranes. For details see the article by Hazen, pages 15527–15531.

♦ Paper of the Week ✕ Author's Choice [S] Online version contains supplemental material [C] Available in online journal only

Human IgG2 Antibodies Display Disulfide-mediated Structural Isoforms*

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In this work, we present studies of the covalent structure of human IgG2 molecules. Detailed analysis showed that recombinant human IgG2 monoclonal antibody could be partially resolved into structurally distinct forms caused by multiple disulfide bond structures. In addition to the presently accepted structure for the human IgG2 subclass, we also found major structures that differ from those documented in the current literature. These novel structural isoforms are defined by the light chain constant domain (C_L) and the heavy chain C_H1 domain covalently linked via disulfide bonds to the hinge region of the molecule. Our results demonstrate the presence of three main types of structures within the human IgG2 subclass, and we have named these structures IgG2-A, -B, and -A/B. IgG2-A is the known classic structure for the IgG2 subclass defined by structurally independent Fab domains and hinge region. IgG2-B is a structure defined by a symmetrical arrangement of a (C_H1-C_L-hinge)₂ complex with both Fab regions covalently linked to the hinge. IgG2-A/B represents an intermediate form, defined by an asymmetrical arrangement involving one Fab arm covalently linked to the hinge through disulfide bonds. The newly discovered structural isoforms are present in native human IgG2 antibodies isolated from myeloma plasma and from normal serum. Furthermore, the isoforms are present in native human IgG2 with either κ or λ light chains, although the ratios differ between the light chain classes. These findings indicate that disulfide structural heterogeneity is a naturally occurring feature of antibodies belonging to the human IgG2 subclass.

The general structural features of human IgG antibodies, including the complete amino acid sequence and disulfide bond pattern for each subtype, were initially established in the 1960s and early 1970s. Antibodies derived from myeloma patients were studied using the diagonal paper electrophoresis method coupled with N-terminal Edman sequencing of peptides, isolated from the proteolytic digestion of the purified antibodies

(1–7). The intra-chain disulfide bonds of the heavy chain (HC)³ and the light chain (LC) were shown to have homologous features in the four IgG subtypes, whereas the inter-chain bridges were found to be characteristically different, as illustrated in Fig. 1 (4). The most distinct differences between the human IgG subclasses are the amino acid composition and structure of the hinge region, including the number and positions of the Cys residues in the hinge (Table 1), which determines the flexibility of the molecule. Another structural difference is the position of the disulfide linkage between the HC and LC. In IgG1, the C-terminal Cys residue of the LC connects to the first Cys residue in the genetic hinge of the HC, whereas in IgG2, IgG3, and IgG4 subtypes, the LC is disulfide-bonded to the Cys residue in the Fab portion of the antibody immediately N-terminal of the CH₁ disulfide loop, which is spatially close in the folded antibody structure (8, 9).

Disulfide connectivities in addition to those originally established for IgGs by Milstein *et al.* (2, 4, 5, 7) have recently been observed in the IgG1 and IgG4 subclasses. For example, the IgG4 subclass has been shown to also form half molecules through stable intra-chain disulfide bonds between the two Cys residues in the hinge (10–12). It has further been demonstrated that *in vivo* exchange between different IgG4 molecules leads to a repertoire of IgG4 molecules with constantly changing Fab domains, which may alter the effect of therapeutic IgG4 molecules (12, 13). Disulfide linkage differences have also been observed in other subclasses. One report (14) suggested that IgG1 is also capable of forming intra-chain disulfide bonds in the core hinge region, although to a far lesser extent than IgG4.

For the IgG2 antibody, the original structural studies, performed on a human myeloma derived-IgG2 with an LC of the λ type, suggested that the structure of the IgG2 is cross-linked by six inter-chain disulfide bonds: two bonds linking each HC to the LC and the other four connecting the two HCs in the hinge region (7). The cystines linking the HC to the LC were for the IgG2 found in similar positions as in the IgG3 and IgG4 mole-

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³ The abbreviations used are: HC, heavy chain; LC, light chain; IgG, immunoglobulin gamma; mAb, monoclonal antibody; CH1, constant region one of heavy chain; CH2, constant region two of heavy chain; CH3, constant region three of heavy chain; C_L, constant region of light chain; Fc, fragment crystallizable; RP-HPLC, reversed-phase high performance liquid chromatography; CE-SDS, capillary electrophoresis sodium dodecyl sulfate; LC/ESI-MS, liquid chromatography electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; NEM, N-ethylmaleimide; CEX, cation exchange; Lys-C, endoproteinase Lys-C; Glu-C, endoproteinase Glu-C; PTH, phenylthiohydantoin.

TABLE 1
Amino acid sequence of the genetic hinge of the human IgG subtypes

IgG subtype	Core hinge sequences
IgG1	EPKSCDKTHTCPPCP
IgG2	ERKCCVECPPCP
IgG3	ELKTPPLGDTHTCPRCP (EPKSCDTPPPCPRCP) ₃
IgG4	ESKYGPPCPSCP

cules (Fig. 1), and the HC-HC disulfide bridges were hypothesized to exist as parallel bonds. These original IgG2 structural studies, which were performed with human myeloma-derived IgG2 with a λ LC, also detected some additional low abundance disulfide-linked peptides that were not further characterized.

Although presently the IgG1 subtype dominates the therapeutic mAbs, the IgG2 subtype may be preferred for certain therapeutic indications due to its greatly reduced effector func-

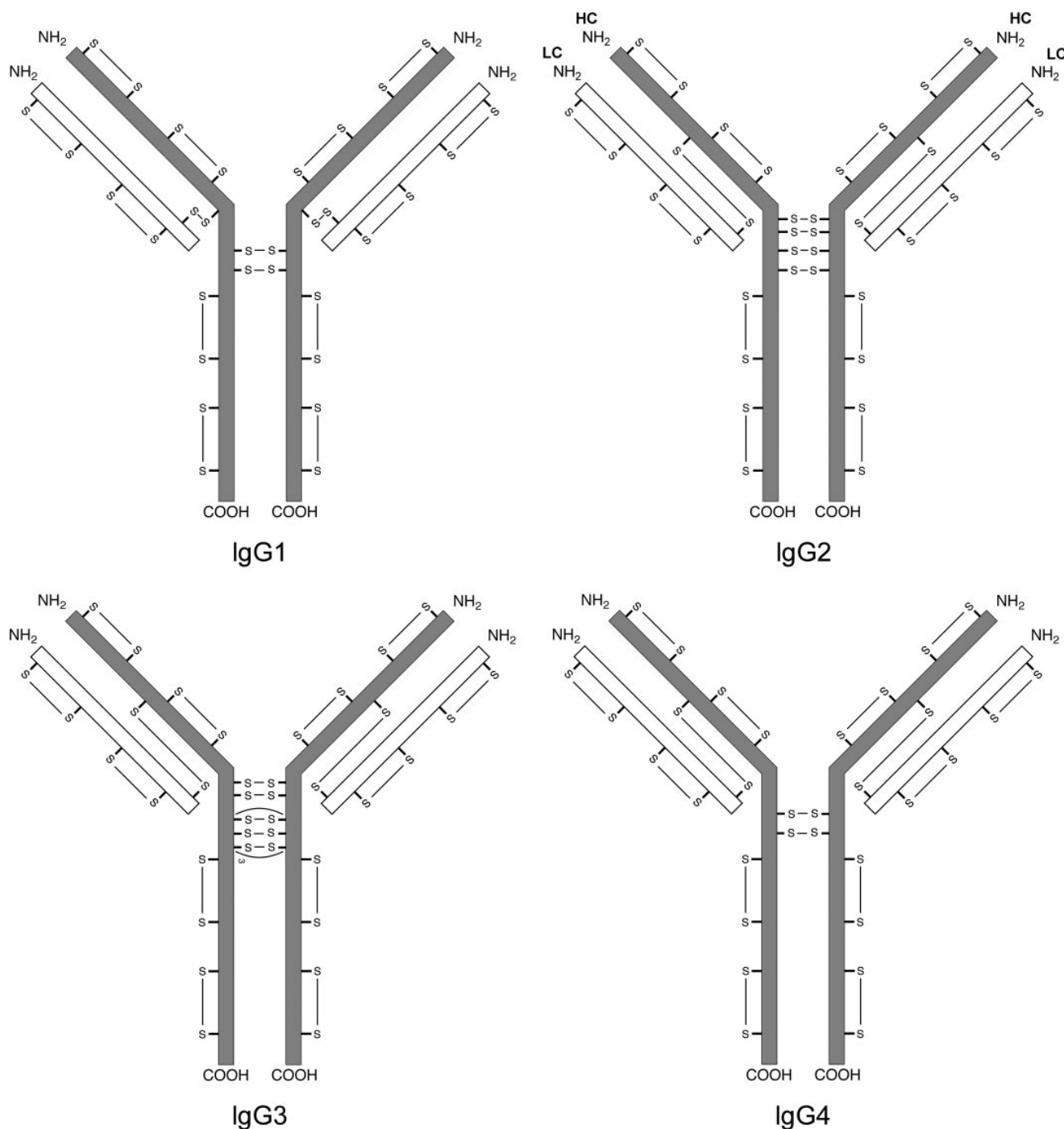


FIGURE 1. Schematic drawings of the human IgG subtypes indicating the originally proposed disulfide connections. IgG1 is the only isotype where the LC was proposed to connect via first Cys residue in the genetic hinge region.

tion activity (15). The scarcity of reported studies of the IgG2 disulfide structure is consistent with its current limited usage in antibody therapeutics. Observations of heterogeneous behavior of IgG2 recombinant mAbs when applying independent separation techniques, CE-SDS, CEX-HPLC, and RP-HPLC under nonreducing conditions, led us to perform detailed structural studies. Here we report in addition to the previously predicted and expected structures of the IgG2 subtype the discovery of heterogeneous disulfide structural isotypes. We extended our structural studies to human myeloma-derived IgG2 with both κ and λ LC as well as to polyclonal IgG purified from normal human serum, and we found that the observed alternate structural isoforms exist in immunoglobulins from natural sources. Our studies provide evidence that the disulfide-mediated structural heterogeneity is an important and intrinsic feature of the IgG2 subclass.

EXPERIMENTAL PROCEDURES

Materials—The recombinant IgG2 monoclonal antibodies used in this study were produced at Amgen Inc. (Thousand Oaks, CA) and consist of two human $\gamma 2$ HCs and two human κ LCs. The antibodies were expressed in Chinese hamster ovary cells and highly purified using well established chromatographic procedures (16). The purified antibodies were analyzed by size-exclusion HPLC and SDS-PAGE to verify purity and integrity. Human IgG2, κ - and human IgG2, λ -purified antibodies derived from plasma of myeloma patients were purchased from Sigma. Human IgG purified from pooled normal human serum was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Guanidine hydrochloride and urea were obtained from ICN Biomedicals Inc. (Aurora, OH). *N*-Ethylmaleimide (NEM), hydroxylamine (NH_2OH) hydrochloride, and tris(hydroxymethyl)aminomethane (Tris) base were obtained from Sigma. Sodium acetate trihydrate, and glacial acetic acid were from J. T. Baker (Phillipsburg, NJ). Acetonitrile was purchased from Burdick and Jackson (Morristown, NJ). Tris(2-carboxyethyl)phosphine solution and trifluoroacetic acid were purchased from Pierce and J. T. Baker. *n*-Propanol was obtained from Burdick & Jackson (Muskegon, MI). Endoproteinase Lys-C (Lys-C) was purchased from Wako Chemicals (Richmond, VA), and endoproteinase Glu-C (Glu-C) was obtained from Roche Diagnostics (Penzberg, Germany).

Nonreduced CE-SDS—The antibody samples were diluted to 5 mg/ml according to the following procedure: 750 μg of antibody solution was combined with 9 μl of 250 mM iodoacetamide resulting in 15 mM iodoacetamide and with the addition of 7.5 μl of the internal standard: benzoic acid at 1 mg/ml to a final concentration of 50 $\mu\text{g}/\text{ml}$. The solution was brought to 150 μl with CE-SDS sample buffer (Bio-Rad). The solution was mixed, centrifuged, and heated at 70 °C in a water bath for 10 min. The mixture was allowed to cool prior to centrifugation at 13,000 rpm for 10 min, and the supernatant was transferred to a polypropylene sample vial for injection. The capillary was pre-conditioned with 0.1 N NaOH for 5 min followed by a water rinse for 5 min and treatment with 0.1 N HCl for 5 min again followed by a 5-min water rinse. The capillary was filled with Bio-Rad CE-SDS run buffer at 6 bars for 3 min before injection

of samples, which were applied to the capillary using an electrokinetic injection at -10 kV for 20 s. The electrophoretic separation took place using -30 kV for 30 min. The resulting electropherograms were obtained by UV detection at 220 nm.

RP-HPLC Analysis—The experimental procedure for analysis of intact antibody was described in detail previously (17, 18). RP-HPLC was performed on an Agilent 1100 HPLC system with a binary pump and a UV detector at 215 or 220 nm. Agilent Zorbax SB300 C8 column (3.5- μm particle size, 300-Å pore size, 1×50 mm), was used for the HPLC analysis. Mobile phase A was 0.1% aqueous trifluoroacetic acid, and mobile phase B contained 90% *n*-propanol, 9.9% water with trifluoroacetic acid.

Preparation of Collected RP-HPLC Fractions for Nonreduced Lys-C Peptide Mapping—Timed fractions from an RP-HPLC run were collected into a solution containing sufficient guanidine HCl to obtain a final concentration of >6 M. Following collection, the samples were concentrated 60-fold using an Amicon-15 centrifugal device then buffer-exchanged with denaturation buffer (6.5 M guanidine hydrochloride, 0.6 mM NEM, 40 mM phosphate, pH 7.0) to a protein concentration of ~ 8 –10 mg/ml prior to Lys-C digestion as described below.

CEX-HPLC Analysis—CEX-HPLC analysis was carried out on an Agilent 1100 HPLC system monitored at 215 nm. A Dionex ProPac WCX-10 (4.0 $\times$ 250 mm) column was operated at ambient temperature. Mobile phase A consisted of 20 mM sodium acetate, pH 5.0, and mobile phase B of 20 mM sodium acetate, 0.5 M NaCl, pH 5.0. Protein was eluted when applying a linear gradient from 20% to 55% B in 70 min at a flow rate of 0.8 ml/min.

Nonreduced Lys-C Enzymatic Digestion and Reduction of Disulfide-linked Peptides—To denature the antibodies under nonreducing condition and perform alkylation of any buried free sulfhydryl groups, 1–3 μl of 0.2 mM–0.6 mM antibody solutions in 20 mM sodium acetate, pH 5.0, was mixed with 7 μl of denaturing buffer consisting of 8 M guanidine hydrochloride, 10 mM NEM, 0.1 M sodium acetate, pH 5.0, and the mixture was incubated at 37 °C for 3 h. When performing the Lys-C digestion on recombinant and native IgG2 antibodies, 100 μg of each antibody was digested under the same conditions. Prior to digestion, the denatured antibody mixture was diluted into 300 μl of digestion buffer consisting of 4 M urea, 20 mM NH_2OH , 0.1 M Tris, pH 7.0. The digestion reaction was carried out by adding 5 μl of Lys-C at 2 mg/ml resulting in an enzyme to substrate ratio of $\sim 1:10$ (w/w) and incubating the mixture at 37 °C overnight. To quench the reaction, trifluoroacetic acid was added to the sample digests to a final concentration of 0.1% (v/v). When reducing the disulfide-linked peptides from the native nonreduced Lys-C peptide digest and thereby creating a digest for a reduced peptide map, Tris(2-carboxyethyl)phosphine was added to a final concentration of 10 mM, and the reduction was performed at room temperature for ~ 30 min prior to the addition of trifluoroacetic acid to a final concentration of 0.1% (v/v).

LC/ESI-MS/MS Analysis of Lys-C Digest—The on-line LC/ESI-MS/MS analyses were carried out using an Agilent 1100 HPLC system coupled with a ThermoFinnigan (San Jose, CA) LCQ or LTQ ion trap mass spectrometer equipped with an electrospray ionization source. The digested antibody was injected onto a Vydac 214TP52 C4 column (5- μm particle size,

300-Å pore size, 2.1×250 mm) set at 60 °C. Mobile phase A was a mixture of water and trifluoroacetic acid in a 1000:1 ratio (v/v), and mobile phase B was a mixture of acetonitrile, water, and trifluoroacetic acid in a 900:100:1 ratio (v/v). Peptides were eluted using a linear gradient of 0–45% B over 90 min (or 0–50% B over 120 min) at a flow rate of 0.2 ml/min and monitored at 214 nm. The solvent peaks were diverted to the waste before the flow entered the ESI source. The ESI source voltage of the LCQ was set at 4.5 kV, and the capillary temperature was set at 220 °C. Mass spectra were acquired from 300 to 2000 m/z in the centroid mode, followed by a data-dependent zoom scan for determining the charge state and a MS/MS scan to determine the sequence of the peptide precursor ion. In the MS/MS scan, the precursor ions were fragmented by collision-induced dissociation with 35% relative collision energy. Peptides were identified using MassAnalyzer, an in-house developed software program, which correlates the experimental tandem mass spectra against the theoretical tandem mass spectra generated of known peptide sequences (19, 20). Masses for large peptides were determined using MagTran, an in-house developed software program according to the ZScore charge deconvolution algorithm (21).

Glu-C Digestion of Peptides Collected from RP-HPLC followed by LC/ESI-MS Analysis—Peptides derived from the Lys-C digest containing multiple disulfide linkages were subjected to Glu-C digestion to further elucidate the connectivity of the cysteine residues. The peptides were manually collected from the RP-HPLC, lyophilized, and reconstituted in 100 μ l of 2 M urea, 20 mM NH_2OH , 100 mM Tris, pH 7.0. Approximately 2 μ g of Glu-C was added to the reconstituted collected peptide, and the digestion was allowed to proceed at 37 °C overnight. The resulting peptides were analyzed by LC/ESI-MS using a similar LC/ESI-MS method as employed for the Lys-C digest with the exception of a steeper gradient of 0–20% B over 5 min followed by 20–45% B over 40 min.

N-terminal Edman Sequencing—Edman degradation was performed on a 494 Procise series protein sequencer (Applied Biosystems, Foster City, CA) using the pulsed-liquid polyvinylidene difluoride method for 10 cycles. Disulfide-linked peptides were collected manually from RP-HPLC and concentrated by SpeedVac before they were loaded onto the polyvinylidene difluoride membrane of the ProSorb cartridge (Applied Biosystems). ~20–100 pmol of peptide was subjected to sequencing at a time. Cystine was identified during the sequencing by the presence of the phenylthiohydantoin (PTH) derivative of cystine in the corresponding cycle (22, 23).

RESULTS

Analytical Separation of the IgG2 mAb—We evaluated the apparent size of the IgG2 mAb by nonreduced CE-SDS, a gel sieving method performed on fully denatured proteins. This technique has the unique capability of resolving immunoglobulins differently depending on their subclasses.⁴ IgG1 molecules generated a single, homogeneous peak, whereas the IgG2 mAb partially resolved into two species (*top panel* of Fig. 2). These results are consistent with our previous report (18), in which

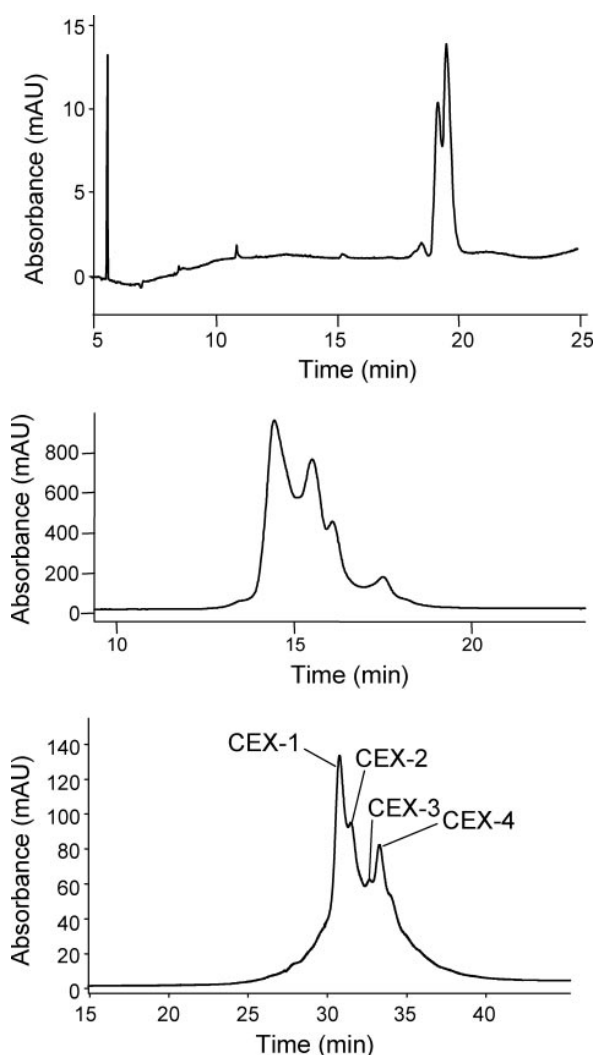


FIGURE 2. Separation of recombinant derived IgG2 by nonreducing CE-SDS (*top*), RP-HPLC (*middle*), and CEX-HPLC (*bottom*).

nonreducing RP-HPLC analysis also revealed IgG2 heterogeneity considered to be disulfide related. An RP-HPLC profile of an IgG2 mAb is shown in the *middle panel* of Fig. 2. In another published study (24), CEX-HPLC of an IgG2 mAb also revealed a complex elution profile. A CEX-HPLC profile of an IgG2 mAb obtained under slightly acidic and nonreducing conditions is displayed in the *lower panel* of Fig. 2. This heterogeneity observation by three different separation techniques could not be explained by variation in glycosylation or differences in primary structure. Previous analysis of human IgG2 by RP-HPLC/MS showed no significant differences between the IgG2 isoforms in molecular mass or glycosylation profiles (18). Additionally, deglycosylation of the IgG2 did not affect the nonreduced CE-SDS profile (data not shown). We also investigated a possibility that downstream processing (16) caused this heterogeneity. Tryptophan oxidation and related changes in structure and retention/migration properties (25) as a cause was rejected, because 1) tryptophan oxidation was not observed by other analytical techniques, including peptide mapping; 2) IgG1 has the same number and positions of tryptophan residues as IgG2

⁴ A. Guo and A. Bolland, unpublished data.

Structural Isoforms of IgG2

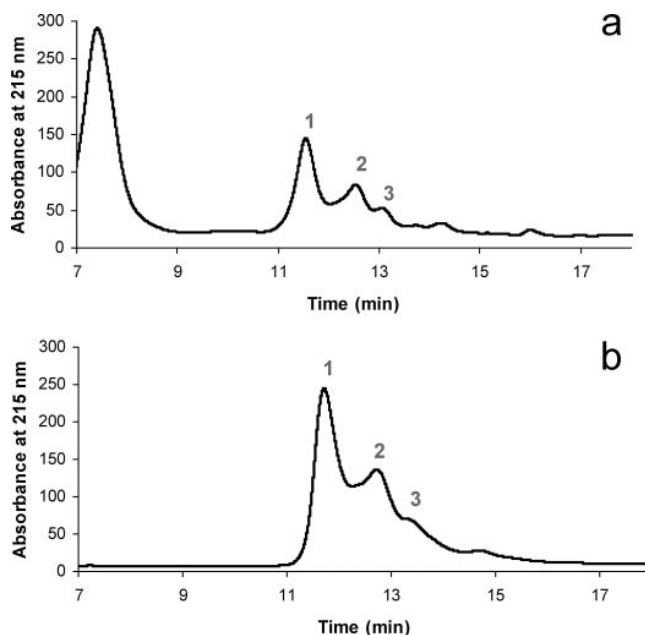


FIGURE 3. RP-HPLC analysis of a human recombinant IgG2 mAb before and after protein A purification. *a*, direct analysis of the IgG2 mAb in cell culture medium after removal of cellular components by centrifugation. The labeled peaks (1–3) containing the IgG2 isoforms were confirmed by direct mass spectral analysis. Additional proteinaceous species were observed during the RP analysis due to host cell contaminants. *b*, analysis of the same IgG2 mAb after protein A purification.

but was homogeneous (26); and 3) the IgG2 antibody samples were kept in the dark and in buffers with minimal levels of free radicals. Alternative folding, induced by an acidic environment (27, 28), and proline isomerization in the Fab (29) were considered as yet other possible reasons for structural heterogeneity of IgG2. In the production and purification processes (16), the recombinant monoclonal antibodies are exposed to an environment where pH is below 4 during elution from the protein A column, during low pH viral inactivation and titration. Other steps in the processing of IgG molecules, such as the viral filtration and the ultrafiltration/diafiltration operation (16) could potentially lead to structural alterations as well. To evaluate a possible role of the downstream processing in the IgG2 structural heterogeneity, the same monoclonal IgG2 material was subjected to RP-HPLC before and after protein A purification. Our results show that the heterogeneous profiles of the IgG2 antibodies are similar before and after purification (Fig. 3), thereby eliminating the downstream processing as a cause of the observed heterogeneity. Observation of the heterogeneity of the IgG2 mAb when applying the described three different separation methods under nonreducing conditions led us to perform detailed structural studies to identify the covalent structural nature of the antibody.

Lys-C Digestion of the IgG2 mAb—The IgG2 disulfide linkages were analyzed by peptide mapping with the endoprotease Lys-C under nonreducing conditions. To cleave the IgG2 mAb under nonreducing conditions, a fairly high guanidine hydrochloride concentration (≥ 5.6 M) was used to disrupt the relatively strong noncovalent interactions (30). Lys-C was chosen as this enzyme cleaves effectively in the presence of a high level

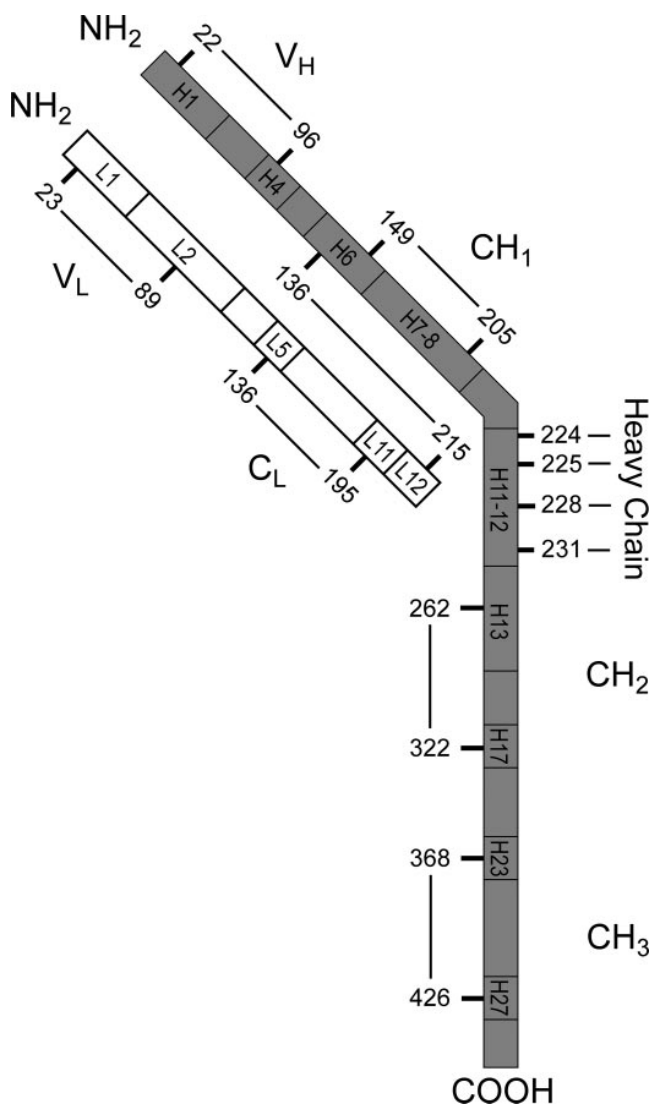


FIGURE 4. Schematic representation of the expected disulfide-containing peptides in the IgG2 mAb obtained by Lys-C digestion under nonreducing conditions. The positions of half-cystines are shown with numbering for this particular antibody molecule. Only half of the molecule is presented. The nomenclature for disulfide bond-containing peptides is as follows: *H1*, heavy chain Lys-C peptide 1; *H7-8*, heavy chain Lys-C peptides 7 and 8, uncleaved due to a Pro residue in the sequence; *H1/H4*, dipeptide connected by one disulfide bond; *(H11-12)₂*, dipeptide connected by four disulfide bonds; and *H6/H7-8/L12*, tripeptide connected by two disulfide bonds.

of denaturant. The denaturing step was performed under mildly acidic conditions (pH 5) in the presence of NEM to block unpaired Cys residues, thereby eliminating any thiol-mediated disulfide exchange (31, 32).

The predicted Cys-containing peptides are illustrated in Fig. 4. The nonreduced and subsequently reduced Lys-C digests of the mAb were analyzed by on-line LC/ESI-MS/MS. The resulting nonreduced and reduced Lys-C peptide maps are shown in Fig. 5 (*top* and *bottom panels*, respectively) and are tabulated in Table 2. Reduced peptides were initially identified by mass, and then confirmed by MS/MS fragmentation patterns. The cystine-containing peptides in the nonreduced map were identified by their disappearance in the reduced map. In peptides

containing a single cystine, the linkage was confirmed by comparing the determined mass to the theoretical combined masses of Cys-containing Lys-C peptides. Assignments of most of these disulfide-containing peptides were also confirmed by their MS/MS fragmentation patterns. For disulfide-bonded peptides containing more than two peptide fragments, the peptides were individually collected from the RP-HPLC and reduced by Tris(2-carboxyethyl)phosphine followed by LC/ESI-MS/MS analysis to identify each peptide fragment. It should be noted that some incomplete cleavages were observed in the digest, due to the presence of Lys-Pro sequences, which are not efficiently cleaved by Lys-C.

Expected Disulfide Structure of the IgG2 mAb—Based on the expected disulfide pattern for the IgG2 mAb, the nonreduced Lys-C digestion should generate seven distinct disulfide-linked peptides, five of them with intra-chain disulfide bonds (H1/H4, H13/H17, H23/H27, L1/L2, and L5/L11), one containing the intra-chain disulfide bond in the CH₁ domain and the HC-LC linkage (H6/H7-8/L12), and one dimeric hinge peptide containing the four inter-heavy chain disulfide bonds (H11-12)₂. All these predicted disulfide-bonded peptides were observed in the nonreduced Lys-C map (labeled as *peaks 1–7* in Fig. 5 and Table 2). Due to incomplete cleavage, low levels of other hinge peptides were also observed ([H10-11-12]₂, *peak 6a*). The *P1* region of the map (defined in Fig. 6 and highlighted in blue in Fig. 5) contains the hinge peptide (H11-12)₂ (*peak 6b*) and H6/H7-8/L12 (*peak 7*). The H6/H7-8/L12 peptide is diagnostic of a Fab arm with the expected linkage between HC and LC and structurally independent from the hinge region as in IgG2-A (see Fig. 8, *top panel*). Upon reduction of the nonreduced Lys-C digest of the mAb, only one Cys-containing peptide (H17) was absent, due to poor chromatographic retention and/or low mass (mass 249.3 Da) in the reduced map.

Certain disulfide connectivities could not be unambiguously assigned using the approach described above. Therefore, those nonreduced peptides were collected from the RP-HPLC and further cleaved with the protease Glu-C. For example,

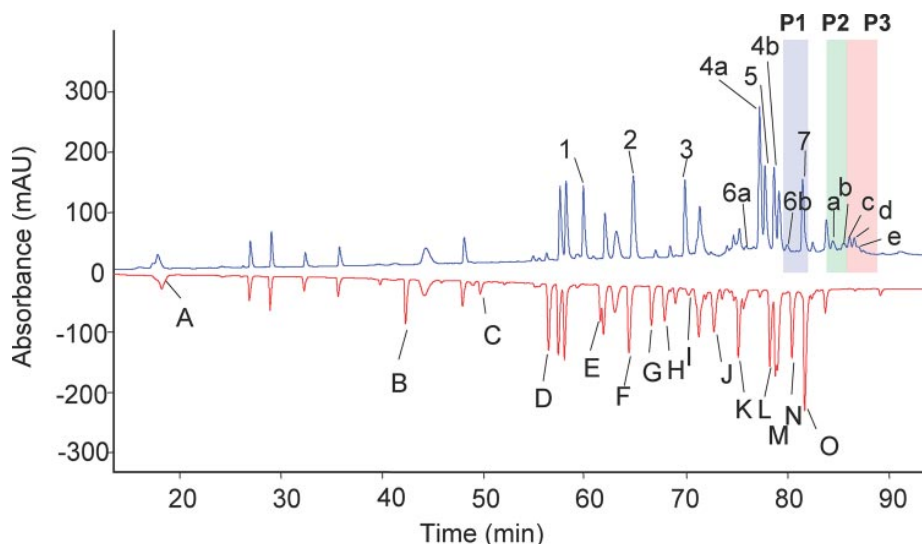


FIGURE 5. LC/ESI-MS/MS analysis of recombinant IgG2 mAb after Lys-C digest. Top trace in blue: nonreduced UV trace. The peaks corresponding to expected disulfide-linked peptides are labeled 1–7 with 4a and 4b as well as 6a and 6b representing incomplete digests at Lys-Pro positions. The peaks corresponding to additional observed disulfide-linked peptides are labeled a–e. Bottom trace in red: reduced UV trace. The peaks corresponding to Cys containing peptides are labeled A–O.

TABLE 2

Determined mass and assignment of peptides with different retention times in the reduced and native Lys-C maps of the mAb (Fig. 5)

Native disulfide-containing peptides				Reduced peptides			
Peak ID	Peptides ^a	Theoretical mass ^b	Observed mass	Peak ID	Peptides	Theoretical mass	Observed mass
		<i>Da</i>	<i>Da</i>			<i>Da</i>	<i>Da</i>
1	H23/H27	4,088.0	4,088.0	A	L12	811.3	811.5
2	L5/L11	3,883.9	3,884.0	B	L11	1,817.9	1,817.8
3	H13/H17	4,803.3	4,803.5	C	H23	1,103.6	1,103.5
4a	L1/L2	11,287	11,286	D	H27	2,986.4	2,986.3
4b	L1-/L2	11,269	11,268	E	H4	2,566.2	2,566.2
5	H1/H4	6,996.9	6,995.5	F	L1	4,436.3	4,436.3
6a	H10-11-12/H10-11-12	6,581.9	6,580.2	G	H6	2,577.3	2,577.2
6b	H11-12/H11-12	5,354.5	5,353.6	H	L5	2,068.0	2,067.9
7	H6/H7-8/L12	10,096	10,094	I	H10-11-12	3,292.7	3,292.5
a	H6/H7-8/H11-12/L12	12,773	12,772	J	H11-12	2,679.3	2,679.4
b	H6/H7-8/H11-12/H10-11-12/L12	16,064	16,062	K	H13	4,556.2	4,556.1
c	H6/H7-8/(H11-12)2/L12	15,451	15,448	L	H1	4,428.2	4,428.5
d	(H6)2/(H7-8)2/(H10-11-12)2/(L12)2	26,774	26,771	M	L2	6,849.5	6,848.1
e	(H6)2/(H7-8)2/H10-11-12/H11-12/(L12)2	26,161	26,157	N	L1-2	11,271	11,270
	(H6)2/(H7-8)2/(H11-12)2/(L12)2	25,547	25,543	O	H7-8	6,709.3	6,709.4
	(H6)2/(H7-8)2/H10-11-12/H10-11/(L12)2	26,549	26,544				

^a H denotes the heavy chain and L the light chain.

^b The numbers in italic represent monoisotopic masses.

Structural Isoforms of IgG2

Glu-C treatment of H6/H7-8/L12 (*peak 7* in Fig. 5 and *top panel* of Fig. 6) cleaved peptide H6 at Glu¹⁴² into H6a of 1634.8 Da and H6b of 962.2 Da. H6a represents the Gly¹²⁷–Glu¹⁴² peptide containing Cys¹³⁶, and H6b represents the Ser¹⁴³–Lys¹⁵² peptide containing Cys¹⁴⁹. The collected peptides and their Glu-C-generated fragments are listed in Table 3. By reducing these complex peptides to ones containing a single cysteine, the connectivities were correctly assigned, and the generation of peptides H6a/L12 (Cys¹³⁶ in the HC to Cys²¹⁵ in the LC) and H6b/H7-8 (Cys¹⁴⁹ to Cys²⁰⁵ in the HC) confirm the existence of the previously predicted and expected HC-LC linkage of the IgG2 molecule as shown in Fig. 4.

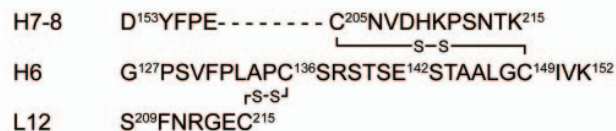
The predicted hinge peptide (H11-12)₂ disulfide linkages were determined by Edman sequencing. PTH-cystines were generated during sequencing cycles 1, 2, 5, and 8 and indicate that all four Cys residues in one HC are disulfide-bonded in parallel to the corresponding Cys residues in the other HC, confirming parallel disulfide bonds in the hinge region of the human IgG2 antibody. The combined approach using nonreduced Lys-C mapping coupled with additional Glu-C mapping of the complex peptide with two cysteine disulfide bonds and the N-terminal sequencing of the hinge peptide has provided data that confirms the presence of the expected structure for IgG2 in the mAb.

Novel Disulfide Structures of the IgG2 mAb—The late eluting peaks, labeled *a–e* in the nonreduced Lys-C map of the mAb

(*top panel* in Fig. 5), disappeared upon reduction, as shown in the reduced Lys-C map (*bottom panel* in Fig. 5), indicating that these peptides contain disulfide bonds. These peaks were individually collected from the nonreduced map and reduced prior to LC/ESI-MS/MS analysis (data not shown). Upon reduction, all these late eluting peaks generated peptides H6, H7-8, and L12 (all belonging to the Fab arm) and hinge peptides H10-11-12, H11-12, or H10-11, although at different ratios. Peptides associated with *peaks a–e*, listed in Table 2, range in mass from ~12 to 28 kDa. *Peaks c–e* (P3 region of reduced map, highlighted in *red* in Fig. 5) are disulfide-linked peptides all containing two copies of Fab arm peptides (H6, H7-8, and L12) and two copies of hinge peptides. The multiple peaks in this region are generated from alternate cleavages in the hinge region. One of the most common linked peptides of the P3 region is shown in the *bottom panel* of Fig. 7. In the associated IgG2 structure, both Fab arms are linked to the hinge region creating a novel linkage pattern, called IgG2-B (illustrated in the *middle panel* of Fig. 8). *Peaks a* and *b* elute in the region of the map marked as *P2* (highlighted in *green* in Fig. 5). The peptides in this region mainly contain one copy of the Fab arm peptides (H6, H7-8, and L12) and two copies of hinge peptides. Because the IgG2 structure predicted from these linked peptides contains only one Fab arm linked to the hinge as in the IgG2-B structure and one Fab arm as in the originally proposed IgG2-A structure, this hybrid structure is referred to as IgG2-A/B (depicted in the *bottom panel* of Fig. 8).

To examine if the CH₁ loop involving Cys¹⁴⁹ and Cys²⁰⁵ in the HC is still intact in the novel structures IgG2-A/B and IgG2-B, *peaks a, b*, and *e* from the P2 and P3 regions of the map were collected individually. Glu-C digestion followed by LC/ESI-MS analysis was performed as described earlier for the expected inter-heavy-light disulfide-bonded peptide H6/H7-8/L12. Table 3 lists the observed disulfide-containing peptides generated from Glu-C digestion of the collected three peaks. The peptide H6b/H7-8 representing the CH₁ loop was detected in all three samples upon digestion with Glu-C, indicating that the intra-chain disulfide bond in the CH₁ domain for all structures is intact as expected. The observation, of the Glu-C peptides H6a/H11-12/L12, H6a/H10-11-12/H11-12/L12, and H6a/(H11-12)₂/L12 derived from the P2 region of the Lys-C map and the Glu-C peptides (H6a)₂/(H11-12)₂/(L12)₂ and (H6a)₂/H10-11/H10-11-12/(L12)₂ derived from the P3 region of the Lys-C map, indicates that Cys¹³⁶ in the HC and Cys²¹⁵ in

Peptide H6/H7-8/L12



Peptide (H11-12)₂

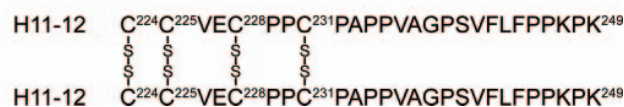


FIGURE 6. Expected disulfide-linked peptides involving inter-chain disulfide bonds. These peptides constitute the P1 group in the nonreduced Lys-C peptide map in Fig. 5.

TABLE 3

Determined average masses and assignment of peptides generated by Glu-C digestion of the peaks 7, a, b, and e collected from the Lys-C map of the mAb

Peak	Mass	Peptide form	Observed Glu-C peptides	Theoretical mass	Observed mass
	Da			Da	Da
7	10,094	H6/H7-8/L12	H6a/L12	2,444.7	2,444.6
			H6b/H7-8	7,669.6	7,668.6
a	12,772	P2	H6a/H11-12/L12	5,122.0	5,121.5
			H6b/H7-8	7,669.6	7,668.6
	16,062	P2	H6a/H10-11-12/H11-12/L12	8,413.0	8,411.0
			H6b/H7-8	7,669.6	7,668.6
b	15,448	P2	H6a/(H11-12) ₂ /L12	7,799.3	7,797.0
			H6b/H7-8	7,669.6	7,668.6
e	25,543	P3	(H6a) ₂ /(H11-12) ₂ /(L12) ₂	10,244	10,244
			H6b/H7-8	7,669.6	7,668.6
	26,544	P3	(H6a) ₂ /H10-11/H10-11-12/(L12) ₂	11,246	11,243
			H6b/H7-8	7,669.6	7,668.6

From P2

H7-8	DYFPEP - - TYTC ²⁰⁵ NVDHKPSNTK
H6	GPSVFPLAPC ¹³⁶ SRSTSESTAALGC ¹⁴⁹ LVK
L12	SFNRGEC ²¹⁵
H11-12	C ²²⁴ C ²²⁵ VEC ²²⁸ PPC ²³¹ PAPPVAGPSVFLFPPKPK
H11-12	C ²²⁴ C ²²⁵ VEC ²²⁸ PPC ²³¹ PAPPVAGPSVFLFPPKPK

From P3

H7-8	DYFPEP - - TYTC ²⁰⁵ NVDHKPSNTK
H6	GPSVFPLAPC ¹³⁶ SRSTSESTAALGC ¹⁴⁹ LVK
L12	SFNRGEC ²¹⁵
H11-12	C ²²⁴ C ²²⁵ VEC ²²⁸ PPC ²³¹ PAPPVAGPSVFLFPPKPK
H11-12	C ²²⁴ C ²²⁵ VEC ²²⁸ PPC ²³¹ PAPPVAGPSVFLFPPKPK
L12	SFNRGEC ²¹⁵
H6	GPSVFPLAPC ¹³⁶ SRSTSESTAALGC ¹⁴⁹ LVK
H7-8	DYFPEP - - TYTC ²⁰⁵ NVDHKPSNTK

FIGURE 7. Schematic of the major variant inter-chain connected peptides of the P2 and P3 groups in the nonreduced Lys-C map in Fig. 5. Peptides within each box are all linked to one another through Cys residues shown in the peptide sequences. Disulfide patterns are not illustrated.

the LC are connected to the hinge via disulfide bonds. The results obtained establish some general disulfide connectivities specific to the IgG2-B and IgG2-A/B structures.

Determining all the specific and exact disulfide connections in the P2 and P3 peptides was challenging due to the closely spaced Cys residues in the hinge region. Some information was gleaned by N-terminal sequencing of peptides H6a/(H11-12)₂/L12 and (H6a)₂/(H11-12)₂/(L12)₂ derived from the Glu-C cleavage. As mentioned earlier, hinge peptide H11-12 contains four cysteine residues at positions 1, 2, 5, and 8. The sequencing data for peptide H6a/(H11-12)₂/L12 shows that the first cycle has no PTH-cystine signal, whereas the cycles 2, 5, and 8 all show PTH-cystine signals. The absence of a PTH-cystine signal in the first cycle suggests that in IgG2-A/B, the two Cys²²⁴ residues are not inter-linked (HC-HC). Rather, the two Cys²²⁴ residues might be disulfide-bonded to Cys²¹⁵ of the LC (HC-LC) and Cys¹³⁶ of the HC (HC-HC). The presence of PTH-cystine in cycles 2, 5, and 8 supports the existence of three parallel disulfide bonds between the two HCs (HC-HC). However, the sequencing data cannot exclude the possible existence of other disulfide arrangements involving intra-chain disulfide loops, including the uncommon Cys-Pro-Pro-Cys and Cys-Cys loops (14, 33) or cross-linking disulfide bridges in the hinge region. Sequencing data for peptide (H6a)₂/(H11-12)₂/(L12)₂ generated by Glu-C digestion and representing IgG2-B shows no PTH-cystine signal at cycles 1 and 2, suggesting no parallel or diagonal disulfide bridges between the first two pairs of Cys

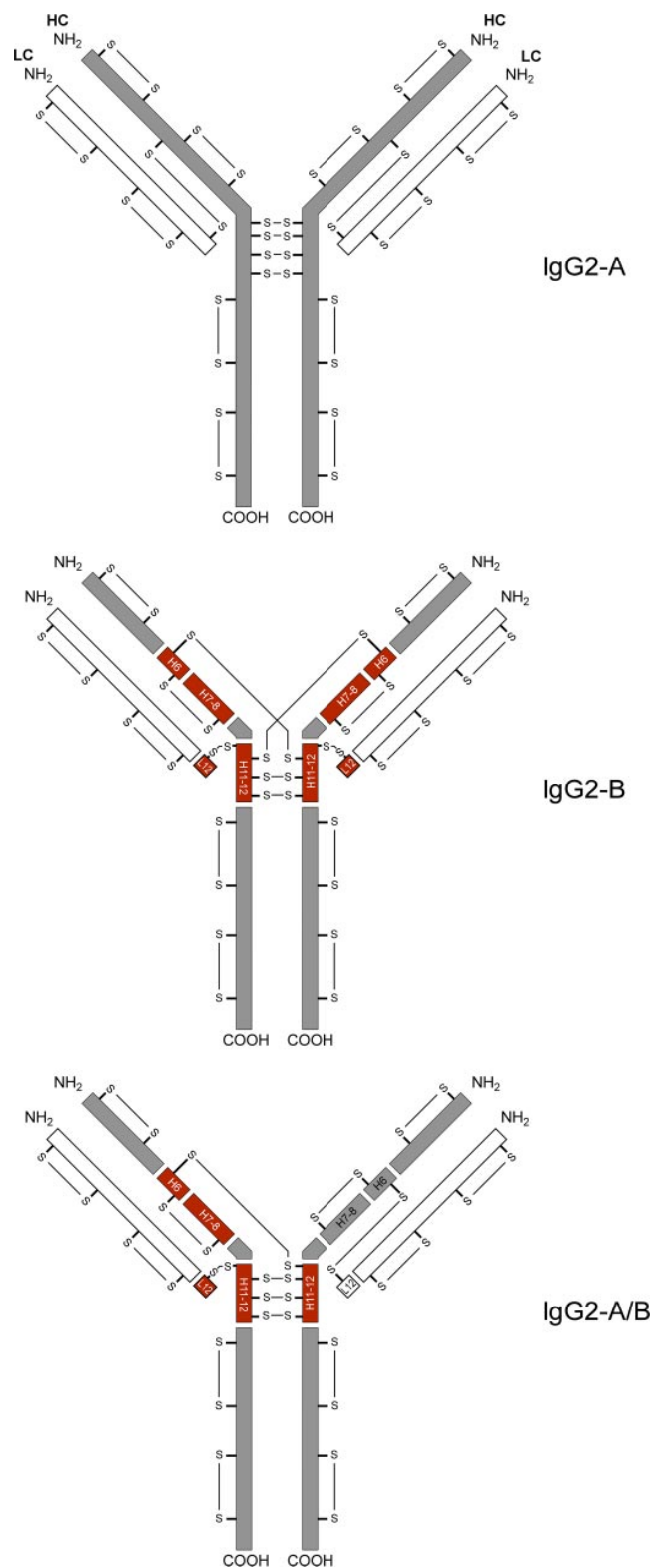


FIGURE 8. Proposed disulfide structural isoforms of human IgG2 antibodies with varying disulfide connections involving the hinge.

residues in the hinge region. This result suggests that the cysteines 224 and 225 are disulfide bonded to either Cys²¹⁵ of LC or Cys¹³⁶ of HC. Both cycles 5 and 8 show significant PTH-cystine

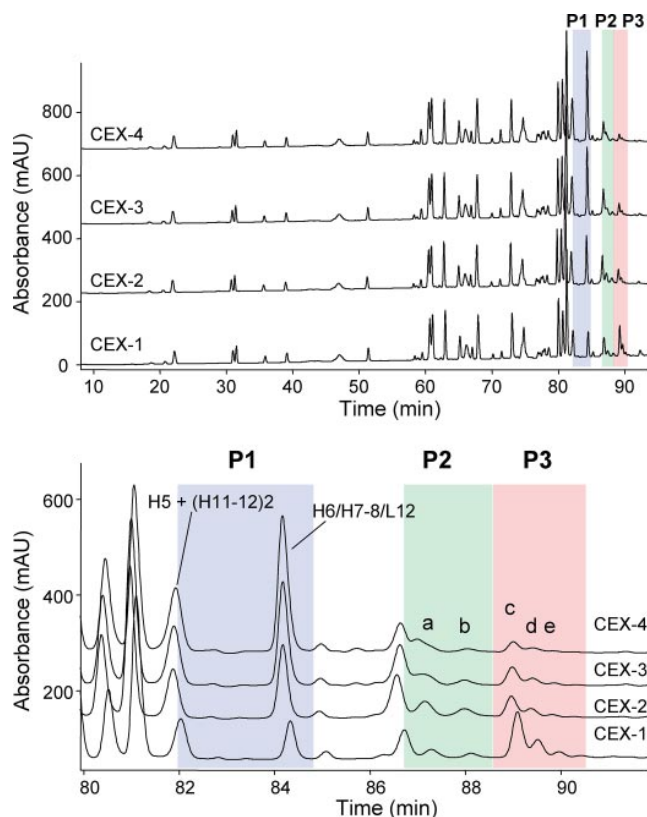


FIGURE 9. *Top panel*: Lys-C peptide maps of collected individual fractions of recombinant mAb separated by CEX-HPLC. Fractions CEX-1, CEX-2, CEX-3, and CEX-4 were collected as indicated in the chromatogram shown in the *bottom panel* of Fig. 2. *Bottom panel*: expanded view of P1, P2, and P3, which shows the positions of the inter-chain disulfide-connected peptides. Note: the peak labeled with (H11-12)₂ contains a co-eluted heavy chain peptide: H5.

signals; therefore, Cys²²⁸ and Cys²³¹ could be interpreted as forming parallel disulfide bridges between the two HCs. As already mentioned, the N-terminal sequencing results cannot exclude other interpretations than that presented above.

Based on LC/ESI-MS/MS and sequencing results of peptides from the P2 and P3 region of the map, a total of three IgG2 structural isoforms is proposed and presented in Fig. 8, as structural isoforms IgG2-A, IgG2-B, and IgG2-A/B, respectively. We are proposing the IgG2-A nomenclature for the previously reported IgG2 structure. Our results confirm the existence of this original structure. The novel IgG2 structural isoform, IgG2-B, has a structure with both of the Fab arms disulfide bonded to the dimeric hinge through Cys¹³⁶ in HCs and Cys²¹⁵ in LCs. The sequencing data support the two in-parallel disulfide bridges involving the last two of the four cysteine residues in the hinge region; however, other possibilities might exist and cannot be excluded based on the sequencing results. A future goal will be to pinpoint the exact linkage of the disulfide bonds in the hinge region. The novel IgG2 structural isoform IgG2-A/B has one Fab arm connected to the dimeric hinge via Cys¹³⁶ in HC and Cys²¹⁵ in LC as in isoform IgG2-B, whereas the other Fab arm has the expected HC-LC disulfide linkage as in isoform IgG2-A. Current data support the three in-parallel disulfide bridges involving the last three of the four Cys residues in the hinge region, but again as previously discussed based on the

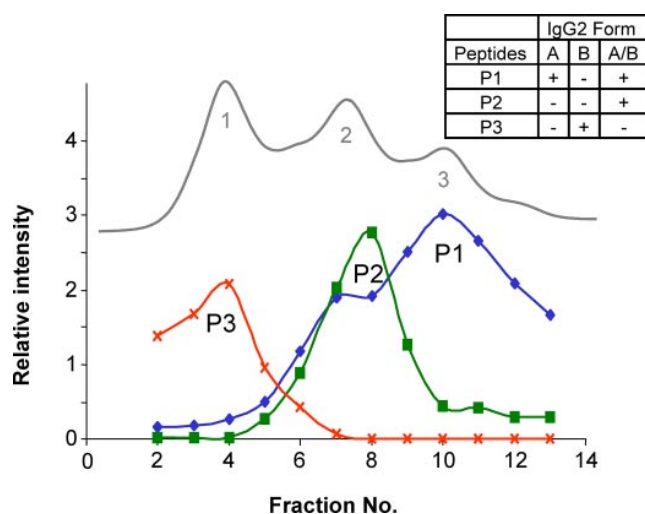


FIGURE 10. **Overlay of RP-HPLC chromatogram (214 nm) peaks (gray) and disulfide form distribution determined by peptide mapping.** The *inset table* details the presence (+) and absence (-) of the three types of peptides in each of the IgG2 disulfide isoforms. (P1 peptides are represented in blue, P2 peptides in green, and P3 peptides in red).

sequencing data, other possibilities such as intra-chain loop or cross-linked disulfide bonds in the hinge cannot be excluded. In summary, the in depth analysis of the large hydrophobic peptide fragments obtained by Lys-C digestion of the IgG2 mAb has revealed novel disulfide-mediated structure isoforms involving the LC, the CH₁, and hinge region of the HC.

Resolved Disulfide Isoforms by CEX-HPLC—CEX-HPLC analysis performed under slightly acidic conditions partially resolves IgG2 mAbs (*bottom panel* of Fig. 2) into multiple species (labeled CEX1, CEX2, CEX3, and CEX4). Individually collected CEX peaks elute, when re-analyzed, at their original position, indicating that under the experimental chromatographic conditions these species are stable with no evidence of interchange.

The four collected CEX-HPLC peaks were analyzed by non-reduced Lys-C peptide mapping (Fig. 9). To compare the relative peak intensities for disulfide-linked peptides, the four chromatograms were normalized using a peptide generated from the LC constant region lacking a Cys residue. The peaks of the P1, P2, and P3 region of the peptide map, representing peptides diagnostic of the IgG2-A, -B, and -A/B disulfide structures, differ in intensity between the CEX fractions. Fig. 9 shows that in CEX-1 P1 is decreased and P3 is increased, whereas in CEX-4 P1 is increased and P3 is decreased, illustrating an enrichment in IgG2-B in the early eluting acidic fractions and in IgG2-A in the late eluting more basic fraction. These results show that the IgG2 structures are stable under nondenaturing conditions and that the chosen nonreduced peptide mapping conditions do not artificially generate the peptides associated with and diagnostic of the structural isoforms of the IgG2 subclass.

Resolved Disulfide Isoforms by RP-HPLC—Peak fractions were also collected from nonreducing RP-HPLC (Fig. 2, *middle panel*) and analyzed by nonreducing Lys-C mapping. After normalizing against a non-Cys-containing peptide, the relative areas of the peaks of the P1, P2, and P3 regions of the peptide map were plotted for each fraction (Fig. 10). The elution profiles of the diagnostic peptides are co-incident with the partially

resolved nonreducing RP-HPLC peaks. *Peak 1*, containing P3, but not P1 and P2, is consistent with the IgG2-B structure; *peak 2*, containing P1 and P2, but no P3, is consistent with the IgG2-A/B structure; and *peak 3*, containing P1 (at twice the level of *peak 2*), but not P2 or P3, is consistent with the IgG2-A structure. These results show that the nonreducing RP-HPLC method is capable of resolving and quantifying the IgG2 disulfide isoforms.

Disulfide-mediated Structural Isoforms of IgG2 Are a General Phenomenon—In addition to the disulfide assignments for the IgG2 mAb described above, disulfide structures of several other human recombinant IgG2 mAbs were verified using the native non-reduced Lys-C mapping technique. Identical disulfide isoforms were observed for all the studied IgG2 mAbs (data not shown).

To show that structural isoforms are a general phenomenon of the IgG2 subtype, we performed nonreduced Lys-C peptide

mapping with LC/ESI-MS on commercial human IgG2 purified from myeloma patients with LC of both the κ and λ types, and on commercial polyclonal human IgG purified from normal human serum. The obtained maps are shown and compared with that of the recombinant mAb described previously (Fig. 11). For the human polyclonal IgG, the Lys-C digest predominantly displays peptides derived from the constant region of the most abundant subtypes, IgG1 and IgG2, whereas the peptides corresponding to the variable regions were not observed due to the low abundance of these very heterogeneous peptides. Mass values associated with *peaks a–e*, peaks from the P2 and P3 region, which are signature peptides for IgG2-A/B and IgG2-B of the peptide map, were detected in human IgG2, κ and normal human serum IgG. The mass values associated with the peptides eluting in the P2 and P3 region of the map of the native human IgG2 antibodies isolated from myeloma plasma and normal human serum are compared with those for the studied recombinant mAb in Table 4. Due to differences in the sequence of the C termini of κ LC and λ LC the expected masses for the variant species in IgG2, λ were recalculated accordingly (the LC peptide corresponding to L12 exhibits a mass of 806.3 Da for the λ LC compared with the mass of 811.3 Da for the κ LC). Fewer and less of the peaks from the P2 and P3 regions of the nonreduced Lys-C map were detected for IgG2, λ , indicating that the IgG2-A/B and IgG2-B structures are less abundant in IgG2 with a λ LC. The data show that the diagnostic peptides P1, P2, and P3 are found in all IgG2 tested, regardless of the origin, supporting the concept of distinct structural isoforms as a general feature of the IgG2 subclass.

DISCUSSION

The data presented here reveal the existence of disulfide structural isoforms in human IgG2, and we use a nomenclature to identify those that are reflective of the symmetry of the forms: IgG2-A, IgG2-B, and IgG2-A/B. Because the human IgG1 hinge region contains disulfides restricted to the hinge, it might be expected that the IgG2 would also contain a disulfide arrangement confined within the hinge region. Our detailed structural characterization has demonstrated that the aforementioned expected structure, here termed IgG2-A, is just one of several disulfide isoforms found in human IgG2. We have demonstrated that human IgG2 forms stable disulfide bonds in which the hinge Cys residues are covalently linked to the C-terminal Cys residue of the LC and a cysteine in the Fab portion (C_{H1} domain) of the HC, forming the basis for the isoforms IgG2-B and IgG2-A/B. The IgG2 structure in which hinge dis-

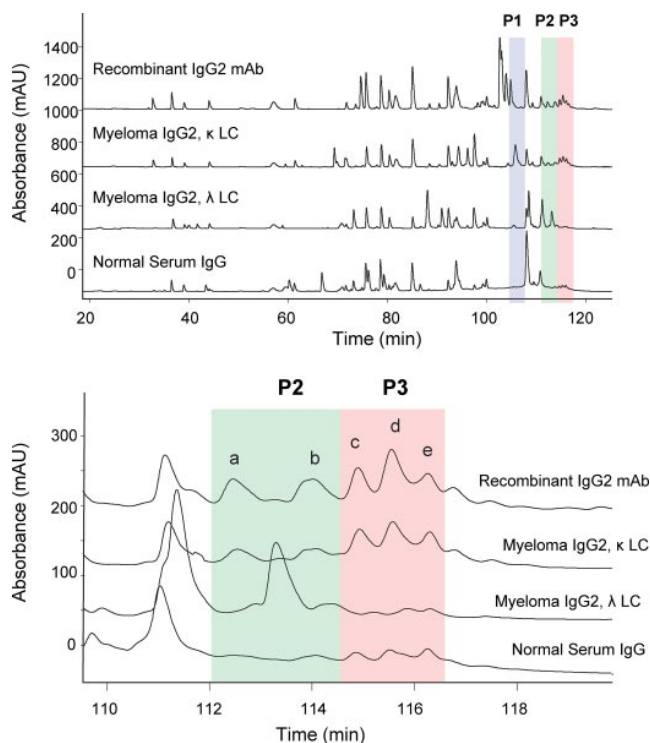


FIGURE 11. *Top panel*: Lys-C peptide maps of recombinant IgG2 mAb, human myeloma IgG2 with κ LC, human myeloma IgG2 with λ LC, and human IgG derived from normal serum. *Bottom panel*: expanded view of the areas P2 and P3, which shows the region of the maps containing the novel inter-chain disulfide connected peptides.

TABLE 4

Determined mass and assignment of P2 and P3 peptides obtained from the native human IgG2 antibodies isolated from myeloma plasma and normal human serum compared to those for the studied recombinant mAb

Peak ID	Peptides ^a	Theoretical mass	Observed mass			
			mAb	Myeloma IgG2 κ LC	Myeloma IgG2 λ LC	Normal serum IgG
		Da	Da	Da	Da	Da
a	H6/H7-8/H11-12/L12	12,773.4	12,772	12,771		12,772
	H6/H7-8/H11-12/H10-11-12/L12	16,064.4	16,062	16,061		16,059
b	H6/H7-8/(H11-12)/L12	15,450.7	15,448		15,443	15,446
c	(H6)2/(H7-8)2/(H10-11-12)2/(L12)2	26,774.3	26,767	26,768	26,762	26,768
d	(H6)2/(H7-8)2/H10-11-12/H11-12/(L12)2	26,160.5	26,155	26,156		26,156
e	(H6)2/(H7-8)2/(H11-12)2/(L12)2	25,547.0	25,543	25,542		25,542

^a H denotes the heavy chain and L and LC the light chain.

ulfides are connected to both Fab arms is termed IgG2-B, whereas the structural hybrid antibody with one independent Fab arm as in IgG2-A and the other Fab arm connected through disulfide bridges to the hinge as in IgG2-B was characterized and is termed IgG2-A/B.

Nonreduced peptide mapping provided the basis for determining the connectivities and provided a semi-quantitative measurement of the relative levels of the disulfide isoforms. Improved quantification can be obtained with low pH cation exchange chromatography and with a nonreducing reversed phase procedure. These methods partially resolve the IgG2-A, IgG2-A/B, and IgG2-B forms as demonstrated in this report. The IgG2 structure reported in an earlier study (7) did not contain these alternate disulfide-linked structures, most likely because those IgG2 structural studies were performed using an IgG2 myeloma antibody with a λ LC. Our studies here show that IgG2 with a λ LC displays far less of the novel structural isoforms than when the antibody has a κ LC, which explains why the IgG2-A structure originally was reported as the single structure of the IgG2 subclass, whereas the IgG2-B and IgG2-A/B structures went undetected in the earlier studies of the IgG2 structure (7). The IgG2-B and IgG2-A/B structures would likely have been discovered and reported in the early structure elucidation of IgG2 if the additional low abundance disulfide-linked peptides, which were noted in the original studies using IgG2 myeloma antibody with the λ LC, had been further characterized.

The presence of the same disulfide bond heterogeneity observed in a human monoclonal antibody of the IgG2 subclass as well as a human myeloma-derived IgG2 strongly suggests that this phenomenon is naturally occurring. That these structures are also observed in IgG from normal human serum significantly strengthens the argument that all the IgG2 structures are a naturally occurring feature of human IgG2.

Early models of immunoglobulin G structure-function envisioned a rather static structure with two functional domains: the antibody binding domains, the Fab, and the Fc domain, which elicits effector functions such as complement dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and antibody clearance via Fc receptors. Since that time, evidence has emerged of a far more dynamic structure with subclass-dependent structural features that provide more complex functionality. The hinge region of the molecule, which was previously thought as a linker between the Fab and Fc domains, appears to be implicated in the elicitation of some of these functional properties (34–36). In the case of IgG2, evidence has been shown that this subclass forms covalent dimers in plasma via disulfide bond formation in the hinge (37); our report provides significant structural detail on the complexity of the disulfide structure of human IgG2. The observation that human IgG2 forms dimers *in vivo* (37) is an intriguing finding that suggests a functional significance of dimerization of the antibody. It is conceivable that the disulfide bond-mediated heterogeneity observed in human IgG2 is related to the dimerization phenomenon, and that, in turn, the rate of dimerization may vary depending on the disulfide bond status of an individual antibody molecule.

Recent reports in the literature have documented that the IgG4 subclass forms intra-chain disulfide bonds in the hinge region, which appear to be in equilibrium with inter-chain disulfide bonds (11), a property that confers monovalency on IgG4 in plasma. In addition, this subclass appears to be bi-specific due to the dynamic exchange of half-molecules (13). Although the mechanism of the exchange reaction resulting in the bi-specific IgG4 is not completely clear, data demonstrate that mixing the IgG4 molecules *in vitro* does not lead to the exchange phenomenon. The IgG4 finding suggests that the exchange reaction is a facilitated process involving a mechanism *in vivo* such as a redox-coupled process and/or binding to another protein such as a soluble or membrane-bound Fc receptor-like molecule. A comparable situation could exist for the IgG2 subclass, and we address the concept of dynamic exchange between the IgG2 structural isoforms in our accompanying report (26).

The functional significance of the disulfide bond heterogeneity is not fully known. With respect to the functional aspects of the phenomenon, the disulfide isoforms can, in principle, impact both effector functions and antigen binding affinity/avidity and thereby lead to some level of physiological control. Although the bioactivity of the IgG2 mAb studied here appears to be comparable for all variant forms (not shown), we demonstrate in the accompanying report (26), that for some IgG2 antibodies, the disulfide forms possess different antigen binding activities. The presence of disulfide isoforms of the IgG2 subclass therefore has the potential to provide a significant level of *in vivo* control of antibody functionality and thereby adds a new dimension to the diversity of the antibody family.

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REFERENCES

1. Pink, J. R. L., and Milstein, C. (1967) *Nature* **214**, 92–94
2. Pink, J. R. L., and Milstein, C. (1967) *Nature* **216**, 941–942
3. Frangione, B., Milstein, C., and Franklin, E. C. (1968) *Biochem. J.* **106**, 15–21
4. Frangione, B., and Milstein, C. (1968) *J. Mol. Biol.* **33**, 893–906
5. Frangione, B., Milstein, C., and Pink, J. R. (1969) *Nature* **221**, 145–148
6. Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., and Waxdal, M. J. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **63**, 78–85
7. Milstein, C., and Frangione, B. (1971) *Biochem. J.* **121**, 217–225
8. Saphire, E. O., Parren, P. W. H. I., Pantphlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R., and Wilson, I. A. (2001) *Science* **293**, 1155–1159
9. Saphire, E. O., Stanfield, R. L., Crispin, M. D. M., Parren, P. W. H. I., Rudd, P. M., Dwek, R. A., Burton, D. R., and Wilson, I. A. (2002) *J. Mol. Biol.* **319**, 9–18
10. Schuurman, J., Van Ree, R., Perdok, G. J., Van Doorn, H. R., Tan, K. Y., and Aalberse, C. (1999) *Immunology* **97**, 693–698
11. Schuurman, J., Perdok, G. J., Gorter, A. D., and Aalberse, R. C. (2001) *Mol. Immunol.* **38**, 1–8
12. Aalberse, R. C., and Schuurman, J. (2002) *Immunology* **105**, 9–19
13. van der Neut Kolfshoten, M., Schuurman, J., Losen, M., Bleeker, W. K., Martínez-Martínez, P., Vermeulen, E., den Bleker, T. H., Wiegman, L., Vink, T., Aarden, L. A., De Baets, M. H., van de Winkel, J. G. J., Aalberse,

- R. C., and Parren, P. W. H. I. (2007) *Science* **317**, 1554–1557
14. Bloom, J. W., Madanat, M. S., Marriott, D., Wong, T., and Chan, S. Y. (1997) *Protein Sci.* **6**, 407–415
15. Canfield, S. M., and Morrison, S. L. (1991) *J. Exp. Med.* **173**, 1483–1491
16. Shukla, A. A., Hubbard, B., Tressel, T., Guhan, S., and Low, D. (2007) *J. Chromatogr. B.* **848**, 28–39
17. Dillon, T. M., Bondarenko, P. V., and Ricci, M. S. (2004) *J. Chromatogr. A* **1053**, 299–305
18. Dillon, T. M., Bondarenko, P. V., Rehder, D. S., Pipes, G. D., Kleemann, G. R., and Ricci, M. S. (2006) *J. Chromatogr. A* **1120**, 112–120
19. Zhang, Z. (2004) *Anal. Chem.* **76**, 3908–3922
20. Zhang, Z. (2005) *Anal. Chem.* **77**, 6364–6373
21. Zhang, Z., and Marshall, A. G. (1998) *J. Am. Soc. Mass. Spectrom.* **9**, 225–233
22. Lu, H. S., Klein, M. L., Everett, R. R., and Lai, P. H. (1986) *Modern Methods in Protein Chemistry*, Plenum, New York, pp. 493–502
23. Marti, T., Rosselet, J., Titani, K., and Walsh, K. A. (1987) *Biochemistry* **26**, 8099–8109
24. Zhang, Y., Goetze, A., Novick, S., Jochheim, C., Boyce, J. M., Gerhart, M., Qin, X., and Gombotz, W. (2003) *Bioprocessing J.* **2**, 37–43
25. Yang, J., Wang, S., Liu, J., and Raghani, A. (2007) *J. Chromatogr. A* **1156**, 174–182
26. Dillon, T. M., Ricci, M. S., Vezina, C., Flynn, G. C., Liu, Y. D., Rehder, D. S., Plant, M., Henkle, B., Li, Y., Deechongkit, S., Varnum, B., Wypych, J., Balland, A., and Bondarenko, P. V. (2008) *J. Biol. Chem.* **283**, 16206–16215
27. Buchner, J., Renner, M., Lilie, H., Hinz, H. J., Jaenicke, R., Kiefhabel, T., and Rudolph, R. (1991) *Biochemistry* **30**, 6922–6929
28. Buchner, J., Rudolph, R., and Lilie, H. (2002) *J. Mol. Chem.* **318**, 829–836
29. Lilie, H., Rudolph, R., and Buchner, J. (1995) *J. Mol. Chem.* **248**, 190–201
30. Dorrington, K. J. (1978) *Can. J. Biochem.* **56**, 1087–1101
31. Bures, E. J., Hui, J. O., Young, Y., Chow, D. T., Katta, V., Rohde, M. F., Zeni, L., Rosenfeld, R. D., Stark, K. L., and Haniu, M. (1998) *Biochemistry* **37**, 12172–12177
32. Yen, T.-Y., Yan, H., and Macher, B. A. (2002) *J. Mass. Spectrom.* **37**, 15–30
33. Creighton, C. J., Reynolds, C. H., Lee, D. H. S., Leo, G. C., and Reitz, A. B. (2001) *J. Am. Chem. Soc.* **123**, 12664–12669
34. Brekke, O. H., Michaelsen, T. E., and Sandlie, I. (1995) *Immunol. Today* **16**, 85–90
35. Dall'Acqua, W. F., Cook, K. E., Damschroder, M. M., Woods, R. M., and Wu, H. (2006) *J. Immunol.* **177**, 1129–1138
36. Redpath, S., Michaelsen, T. E., Sandlie, I., and Clark, M. R. (1998) *Hum. Immunol.* **59**, 720–727
37. Yoo, E. M., Wims, L. A., Chan, L. A., and Morrison, S. L. (2003) *J. Immunol.* **170**, 3134–3138